A Constitutively Activated Chimeric Cytokine Receptor Confers Factor-Independent Growth in Hematopoietic Cell Lines

By Yayoi Shikama, Dwayne L. Barber, Alan D. D'Andrea, and Colin A. Sieff

The high-affinity receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF) comprises at least 2 distinct subunits, α and β common (β2), whereas the normal erythropoietin receptor (nEpoR) comprises only one known subunit. An arginine to cysteine (R129C) mutation of the extracytoplasmic domain of the murine EpoR leads to Epo-independent growth in transfected cells (cEpoR). To investigate the proliferative functions of the cytoplasmic regions of each GM-CSF subunit separately and the potential of the R129C EpoR mutation to induce factor-independent growth through heterologous receptor regions, we constructed four hybrid receptors: the extracellular region of either murine nEpoR or cEpoR linked to the transmembrane and cytoplasmic regions of either the human GMα or β subunit (nExn, nExβ, cExα, and cExβ). We then expressed them in an interleukin-3-dependent murine cell line (Ba/F3). Expression of nEx led to Epo-dependent growth, whereas expression of cExβ conferred factor-independent growth. Surprisingly, expression of cExα also resulted in factor-independent cell growth, whereas nExα did not respond to Epo. Furthermore, the functional hybrid receptors showed Epo-dependent (nExβ) or constitutive (cExα and cExβ) tyrosine phosphorylation of the cytoplasmic kinases JAK1 and JAK2. We reasoned that the proliferative signal of cExα was transduced either through the α tail itself or through an accessory protein such as the endogenous murine β common subunit (μβα). To distinguish these possibilities, the chimeric receptor cExα was expressed in the interleukin-2-dependent murine cell line, CTLL-2, that does not express μβα. cExα did not induce cell growth in CTLL-2; however, when μβα was coexpressed with cExα in CTLL-2, factor-independent growth was reconstituted. In conclusion, the cytoplasmic domain of the GMα subunit requires a β chain for transduction of a proliferative signal. Furthermore, the R129C EpoR mutation can constitutively activate heterologous receptors to mediate factor-independent proliferation.

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MATERIALS AND METHODS

Cells and cell culture. The IL-3-dependent mouse hematopoietic cell line (Ba/F3) and the IL-2-dependent T-cell line (CTLL-2) were maintained in RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) newborn calf serum (Sigma, St Louis, MO) and 500 pmol/L of murine IL-3 (mIL-3; kindly provided by Immune, Seattle, WA) or 2 U/ml of mIL-2 (Boehringer Mannheim Corp, Indianapolis, IN) and 2-mercaptoethanol (Sigma), respectively.

Hybrid and full-length receptor construction. The cDNAs for the murine nEpoR and cEpoR were digested at the NheI site, located 6 codons upstream from the boundary between the extracellular and transmembrane regions. The 3’ cDNA fragments of the human...
GMRA and β subunits, encoding 5 amino acid residues of the extracellular region, the transmembrane region, and the cytoplasmic regions, were obtained by polymerase chain reaction (PCR), creating a Nhe I site at the 5' end, and were sequenced to confirm their fidelity. The digested 5' fragments were ligated to the PCR products. The resulting constructs were ligated into the eukaryotic expression vector, pMT21 (Fig 1). The full-length human IL-3Ra, GMRA, and β subunits were electroporated into Ba/F3 cells, as previously described.21,22

Transfected cell lines. Ba/F3 or CTLL-2 cells were washed twice and resuspended in cold phosphate-buffered saline (PBS) at a final concentration of 1 x 10^6/mL. The linearized cDNAs of the receptors along with the neomycin resistance gene were added to the cell suspension. After the cells were placed on ice for 10 minutes, the electroporation was performed at 960 V and 270 mA with the time constant set by the apparatus (Bio-Rad, Richmond, CA). The electroporated Ba/F3 or CTLL-2 cells were allowed to recover for 2 to 3 days in the presence of mL-3 or mL-2, respectively, and were then selected for neomycin resistance in G418. The CTLL-2 transfecteds with nEa and cEa were electroporated with mEaβ, (a gift from Dr A. Miyajima, University of Tokyo, Tokyo, Japan) along with the hygromycin resistance gene. These cells were selected in 2 U of mL-2 and 0.3 mg/mL of hygromycin (Calbiochem, La Jolla, CA). Four to five weeks later, all cells were cultured in 5 U/mL Epo, no growth factor, or (3418 plus mIL-3 or mIL-2.

Metabolic labeling. Parental Ba/F3 and transfecteds were washed and incubated in methionine- and cysteine-free RPMI 1640/5% FCS for 2 minutes, after which 400 μCi/mL 35S methionine/ cysteine (New England Nuclear, Boston, MA) was added and the cells were incubated for a further 5 to 6 hours at 37°C. The metabolically labeled cells were washed twice in PBS and solubilized in lysis buffer that contained 1% NP-40 (Sigma), 50 mmol/L Tris (pH 8.0) with protease inhibitors (10 mg/mL leupeptin, 10 mg/mL pepstatin, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.2 U/mL aprotinin). After centrifugation (160,000g for 15 minutes at 4°C), the supernatants were incubated with a 1:100 dilution of rabbit antiserum against Epo receiving N-termnus amine23 or control serum for 4 hours or overnight at 4°C. Protein A Sepharose beads (15 μL packed volume) were then added and, after 1.5 hours at 4°C, the beads were washed four times in lysis buffer. The bound complex was eluted by boiling for 5 min in 40 μL Laemmli sample buffer containing 5% 2-mercaptoethanol. The sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5%) and autoradiography. Exposure times were 1 to 48 hours.

Immunoprecipitation and immunoblot analysis. Ba/F3 socalones that expressed various hybrid receptors were incubated in RPMI 1640-1 ng/mL bovine serum albumin (BSA) for 8 hours. Cells were stimulated with no added factor, 50 U/mL IL-3, or 50 U/mL Epo. Lysates were prepared as described by lysing in 1% Triton X-100 or 1% Brij 96. The protein concentration of each lysate fraction was then determined. Two milligrams of each fraction was immunoprecipitated with either JAK1 (catalogue no. 06-272) or JAK2 (catalogue no. 06-255) peptide-specific antibodies (Upstate Biotechnology Inc, Lake Placid, NY). After SDS-PAGE and transfer to nitrocellulose, the membrane was incubated with 1 mg/L 4G10 monoclonal antiphosphotyrosine antibody, followed by peroxidase (HRP)-sheep antimouse lgG.*' After enhanced chemiluminescence (ECL) detection (Amersham, Arlington Heights, IL), the blot was stripped and reprobed with a peptide-specific antibody for JAK1 or JAK2 followed by HRP-protein A and ECL detection.

For the Epo receptor immunoprecipitation/4G10 or JAK Westerns, an amino-terminal antipeptide Epo receptor antibody25 or a polyclonal antipeptide antibody raised against a GST fusion protein corresponding to residues 1 through 225 of the murine Epo receptor was used. Lysates were prepared in 1% Brij 96 and immunoprecipitations were performed. Western analysis was performed as described above.

In vitro kinase assay. Immune complexes were isolated as described above. Samples were washed twice in 50 mmol/L Tris-HCl (pH 8.0)-150 mmol/L NaCl-0.1% Brij 96 and once in kinase buffer (20 mmol/L N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid [HEPES; pH 7.4], 50 mmol/L NaCl, 5 mmol/L MgCl2, 5 mmol/L MnCl2, 100 mmol/L Na3VO4) as described previously.26 Kinase reactions were performed for 15 minutes at room temperature (250

**Fig 1. Schematic representation of EpoR/GM-CSFR hybrid receptors.** Normal GMRA, β subunit, and both native (n) and constitutive (c) EpoR are shown on the left. The brackets with arrows indicate the contribution of the extracellular regions of nEpoR and cEpoR to the hybrid receptors, along with the cytoplasmic regions of GMRA or β. Products, Wilmington, DE). The 125I-Epo had a specific activity of 1.78 × 10^18 cpm/mol. Before the incubation with 125I-Epo, all cells were cultured for 4 hours or more in Epo-free media containing 10% newborn calf serum. A total of 1 to 5 x 10^6 cells were washed and then resuspended in binding media, RPMI 1640 with 4% fetal calf serum (FCS), 30 mmol/L HEPES (pH 7.4), and 0.04% sodium azide that contained the indicated concentrations of radiolabeled Epo incubated at 4°C for 18 hours. The cells were then centrifuged through a 3:2 mixture of dibutyl phthalate (Sigma)-bis-phthalate oil (Eastman Kodak, Rochester, NY). The cell-associated radioactivity of the pellets was counted in a gamma counter (Packard Instrument Co, Meriden, CT). Nonspecific binding was determined by counting the cell pellets from cells that were incubated with each concentration of 125I-Epo in the presence of 100-fold excess of unlabeled Epo. The binding data were subjected to Scatchard analysis using the LIGAND program.20

Metabolic labeling. Parental Ba/F3 and transfecteds were washed and incubated in methionine- and cysteine-free RPMI 1640/5% FCS for 30 minutes, after which 400 μCi/mL 35S methionine/cysteine (New England Nuclear, Boston, MA) was added and the cells were incubated for a further 5 to 6 hours at 37°C. The metabolically labeled cells were washed twice in PBS and solubilized in lysis buffer that contained 1% NP-40 (Sigma), 150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0) with protease inhibitors (10 mg/mL leupeptin, 10 mg/mL pepstatin, 2 mmol/L phenylmethylsulfonyl fluoride, and 0.2 U/mL aprotinin). After centrifugation (160,000g for 15 minutes at 4°C), the supernatants were incubated with a 1:100 dilution of rabbit antimurine Epo N-terminus antisem23 or control serum for 4 hours or overnight at 4°C. Protein A Sepharose beads (15 μL packed volume) were then added and, after 1.5 hours at 4°C, the beads were washed four times in lysis buffer. The bound complex was eluted by boiling for 5 min in 40 μL Laemmli sample buffer containing 5% 2-mercaptoethanol. The sample was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 7.5%) and autoradiography. Exposure times were 1 to 48 hours.

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\textbf{RESULTS}

The neβ hybrid receptor confers Epo-dependent growth, whereas cEβ and cEα confer factor-independent growth in Ba/F3 cells. Cell surface expression of the hybrid receptors was first assessed by FACS analysis. Transfected cells were analyzed with a purified rabbit polyclonal antibody against the EpoR N-terminus that reacts with both the native EpoR and the constitutively active EpoR (Fig 2A). All hybrid receptors were efficiently expressed on the surface of transfected Ba/F3 cells. To confirm the size of the expressed receptors, immunoprecipitation was performed using rabbit antiserum against EpoR N-terminus (human β),\textsuperscript{21} and antiserum raised to peptides at the carboxy-terminus of human β,\textsuperscript{2} The precipitated samples were separated on SDS-PAGE (7.5%) and transferred onto a nitrocellulose membrane. The membrane was blocked by 5% BSA (Sigma) in Tris-buffered saline (TBS; 10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl) and incubated with HRP-streptavidin (1:10,000) in TBS with Tween 20 (TBST) for 1 hour. After washing with TBST eight times, immunoreactive proteins were detected by ECL.

JAK1 and JAK2 kinases are activated by the IL-3R. The above data suggest that β induces tyrosine phosphorylation of JAK1 and JAK2, whereas the EpoR, as expected, only induces phosphorylation of JAK2. To determine whether full-length receptors showed a similar pattern of JAK phosphorylation and also whether this correlated with JAK activation, we performed in vitro kinase assays on Ba/F3 cells that stably expressed EpoR, huIL-3Rα + β, and huGMα + β. Ligand-induced tyrosine phosphorylation patterns of JAK1 and JAK2 agreed with the results obtained with the hybrid receptors, i.e., JAK1 was phosphorylated in cell lines responsive to huIL-3 and huGM-CSF only, whereas JAK2 was phosphorylated in all three cell lines. In vitro kinase assays are shown in Fig 5. Murine IL-3 induced in vitro

\textbf{Thymidine incorporation assay.} Ba/F3 or CTLL-2 wild-type and transfectants were washed three times and plated at 3 to 5 \times 10^5 cells/well in duplicate in 200 µL of appropriate media in microtiter wells. The microtiter plates were incubated for 72 hours at 37°C in a 5% CO2 incubator as previously described.\textsuperscript{4} Four hours before harvest, 1 mCi of [H]thymidine (New England Nuclear) was added to each well and incubated at 37°C in 5% CO2. Cells were harvested on filter papers by an automated harvester (Skatron, Traby, Norway) and counted in a β-scintillation counter (Wallac, Gaithersburg, MD).

\textbf{Biotinylation.} The cells were washed three times and resuspended in cold PBS containing 1 mg/mL bovine serum albumin (Pierce, Rockford, IL) at a cell concentration of 5 \times 10^6/mL. After rotation in foil at 4°C for 45 minutes, the biotinylated cells were washed four times in cold PBS with 0.15% (wt/vol) glycine (Sigma) and solubilized in 1% NP-40 lysis buffer, and the samples were subjected to immunoprecipitation (as described above) with a polyclonal antibody to Raf-1 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-EpoR (amino-terminus),\textsuperscript{21} and antisera raised to peptides at the carboxy-terminus of human β,\textsuperscript{2} The precipitated samples were separated on SDS-PAGE (7.5%) and transferred onto a nitrocellulose membrane. The membrane was blocked by 5% BSA (Sigma) in Tris-buffered saline (TBS; 10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl) and incubated with HRP-streptavidin (1:10,000) in TBS with Tween 20 (TBST) for 1 hour. After washing with TBST eight times, immunoreactive proteins were detected by ECL.

\textbf{Functional EpoR/GMα hybrid receptors phosphorylate JAK1 and JAK2.} Previous studies have shown that IL-3,\textsuperscript{24} GM-CSF,\textsuperscript{25} IL-5,\textsuperscript{26} and Epo\textsuperscript{22,27} activate the cytoplasmic tyrosine kinase, JAK2. To test for JAK phosphorylation, Ba/F3 subclones expressing various hybrid receptors were stimulated with either IL-3 or Epo, and an immunoprecipitation with a peptide-specific JAK1 antibody was performed (Fig 4A). Immunoblotting with anti-phosphotyrosine showed that IL-3, but not Epo, induced JAK1 tyrosine phosphorylation in Ba/F3 cells that expressed nEpoR, nEα, cEpoR, and cEα. Interestingly, both Epo and IL-3 induced JAK1 phosphorylation in Ba/F3-nEβ (lanes 8 and 9). Stripping and reprobing the immunoblot with a peptide-specific JAK1 antibody indicated that equivalent amounts of JAK1 had been immunoprecipitated. Taken together, these results show that muβ induces JAK1 phosphorylation in Ba/F3 cells. The cEβ receptor also showed Epo-dependent induction of JAK1 (lane 18), suggesting that the cytoplasmic region of the hybrid receptor may determine which JAK is activated. Consistent with these results, there is a low level of JAK1 tyrosine phosphorylation in cEα and cEβ (lanes 13 and 16) in the absence of stimulation, but not in cEpo (lane 10), which does not display Epo-dependent JAK1 phosphorylation.
kinase activation of JAK1 in parental Ba/F3 (lane 2) and in cells that expressed huIL-3R (lane 6), huGMR (lane 9), and EpoR (lane 12). JAK1 was also activated by huIL-3 in Ba/F3-huIL-3R (lane 7), but, somewhat surprisingly, not by huGM-CSF in Ba/F3-huGMR cells (lane 10). Epo failed to activate JAK1, as previously reported. As expected, JAK2 was activated by muIL-3 in all cell lines (lanes 2, 6, 9, and 12) and by huIL-3 (lane 7), huGM-CSF (lane 10), and Epo (lane 13). These results confirm that \( \beta \) signals are associated with both tyrosine phosphorylation and kinase activation of JAK1 and JAK2.

cE\( \alpha \) mediates constitutive growth through cooperation with the mu\( \beta \) polypeptide. To test the possibility of the involvement of mu\( \beta \) in the constitutive growth of Ba/F3-
cEα cells, the hybrid receptors were expressed in the murine IL-2–dependent cell line CTLL-2. CTLL-2, unlike the Ba/F3 cell line, do not express muβc. The ectopic receptors were expressed on the cell surface (Fig 6). The growth response of each transfectant was analyzed by ³H-thymidine assay. Ba/F3-nEpoR and Ba/F3-nEβ responded to Epo in a dose-dependent manner. The cEpoR and cEβ polypeptides transduced a proliferative signal in the absence of Epo, whereas nEα did not proliferate in Epo at all (data not shown). These were the same proliferation patterns observed in Ba/F3 (Fig 3). In contrast, CTLL-cEα cells did not proliferate in the absence of IL-2 (Table 2). The discrepancy in the growth pattern between Ba/F3-cEα and CTLL-cEα suggested that murine βc was involved in signal transduction in the Ba/F3-cEα cells.

To determine whether the introduction of human or murine βc into CTLL-2 transfectants could reconstitute factor-independent growth, the cDNAs for human or murine βc were transfected into both CTLL-nEα and CTLL-cEα cells. Human βc transfectants did not reconstitute Epo responsiveness in CTLL-nEα cells, and factor-independent growth was not conferred in CTLL-cEα cells (data not shown). To ensure that βc was expressed appropriately, proteins expressed on the cell surface were labeled with biotin. Solubilized cellular proteins were then immunoprecipitated with antibodies to βc, transferred to nitrocellulose, and probed with labeled streptavidin. The results showed that biotinylated 140-kD human βc was expressed on the cell surface of the CTLL-nEα and CTLL-cEα cells (Fig 7, lanes 6 and 12). Hybrid nEα and cEα receptors were also expressed on the cell surface (lanes 2, 5, 8, and 11). As a negative control, Raf-1, a cytoplasmic protein, was not biotinylated and therefore not detected (lanes 1, 4, 7, and 10).

We next tested the growth characteristics of the various CTLL cells transfected with muβc (Table 2). Although all the transfected cells remained responsive to IL-2, CTLL-cEα+muβc cells were factor-independent and proliferated to a similar degree in no growth factor or in Epo, in marked contrast to the cells that expressed cEα alone, which died. Cells that coexpressed muβc and nEα remained unresponsive to Epo, and transduction of muβc alone did not result in factor-independent growth (data not shown). These results indicated that muβc functionally interacts with the hybrid receptor cEα to induce factor-independent cell growth.

### Table 1. Number of Expressed Receptors and Binding Affinity

<table>
<thead>
<tr>
<th>Expressed Receptor</th>
<th>Affinity (pmol/L)</th>
<th>Binding Sites (per cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nEpoR</td>
<td>467</td>
<td>1,790</td>
</tr>
<tr>
<td>nEα</td>
<td>482</td>
<td>5,670</td>
</tr>
<tr>
<td>nEβ</td>
<td>54</td>
<td>2,679</td>
</tr>
<tr>
<td>cEpoR</td>
<td>325</td>
<td>2,856</td>
</tr>
<tr>
<td>cEα</td>
<td>55</td>
<td>4,088</td>
</tr>
<tr>
<td>cEβ</td>
<td>62</td>
<td>1,219</td>
</tr>
</tbody>
</table>

Fig 3. Growth characteristics of Ba/F3 transfected with muβc. Although all the transfected cells remained responsive to IL-2, CTLL-cEα+muβc cells were factor-independent and proliferated to a similar degree in no growth factor or in Epo, in marked contrast to the cells that expressed cEα alone, which died. Cells that coexpressed muβc and nEα remained unresponsive to Epo, and transduction of muβc alone did not result in factor-independent growth (data not shown). These results indicated that muβc functionally interacts with the hybrid receptor cEα to induce factor-independent cell growth.
A

**Fig 5.**  \( \beta \) activates JAK1 and JAK2. Various Ba/F3 cell lines expressing the cytokine receptor indicated were incubated in the absence of cytokine under serum-free conditions for 8 hours and stimulated with 100 ng/mL murine IL-3, 100 ng/mL human IL-3, 100 ng/mL human GM-CSF, or 50 U/mL human EPO for 10 minutes. Cells were lysed and equivalent amounts of cell lysates were subjected to immunoprecipitation with a JAK1 peptide-specific antibody (upper panel) or JAK2 peptide-specific antibody (lower panel). After washing the immunoprecipitates, an in vitro kinase reaction was performed as described in the Materials and Methods. The phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. The positions of phosphorylated JAK1 and JAK2 are indicated by arrows.
CHIMERIC RECEPTORS AND FACTOR INDEPENDENCE

Table 2. \( ^{3} \text{H}-\text{Thymidine Incorporation of CTLL Transfectants} \)

<table>
<thead>
<tr>
<th>Cell</th>
<th>Growth in IL-2*</th>
<th>Growth in Epo</th>
<th>Nil</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTLL (wild-type)</td>
<td>135,481 ± 1,715</td>
<td>195 ± 123</td>
<td>69 ± 10</td>
</tr>
<tr>
<td>CTLL-cEa (exp 1)</td>
<td>69,700 ± 128</td>
<td>195 ± 101</td>
<td>184 ± 361</td>
</tr>
<tr>
<td>CTLL-cEa (exp 2)</td>
<td>72,700 ± 1,780</td>
<td>195 ± 161</td>
<td>201 ± 251</td>
</tr>
<tr>
<td>CTLL-cEa (exp 1) + mu( \beta )</td>
<td>125,000 ± 6,600</td>
<td>36,300 ± 2,700</td>
<td>31,500 ± 1,000*</td>
</tr>
<tr>
<td>CTLL-cEa (exp 2) + mu( \beta )</td>
<td>126,000 ± 6,300</td>
<td>16,600 ± 6,500*</td>
<td>17,400 ± 728*</td>
</tr>
</tbody>
</table>

* Wild-type or transfected CTLL cells were grown in IL-2 (3 nmol/L), Epo (4.5 U/mL), or medium alone (nil) for 72 hours as described (see Materials and Methods).
† Cells did not survive in Epo or no factor.
‡ Factor-independent growth.

DISCUSSION

The novel findings in this study are that the constitutive R129C extracellular domain of the EpoR can confer factor-independent growth not only through direct activation of a heterologous signaling competent cytoplasmic domain (cE\( \beta \)), but also indirectly through mu\( \beta \) (cE\( \alpha \)). Secondly, we show that both JAK1 and JAK2 are activated by receptors that signal through \( \beta \)c.

In this study, a ligand-induced proliferative signal was mediated through nEpoR and nE\( \beta \) in both Ba/F3 and CTLL-2. The R129C mutation (cE\( \beta \)) converted the factor-dependent growth of nE\( \beta \) to constitutive proliferation. These results agree with the hypothesis that homodimerization of a signaling competent component is sufficient for signal transduction. However, although nE\( \alpha \) did not transduce a growth signal, surprisingly, cE\( \alpha \) led to constitutive Ba/F3 growth.

The proliferative function of the GMR\( \alpha \) subunit has been controversial. Three isoforms of the human \( \alpha \) subunit have been isolated: a soluble form\(^{3}\); GMR\( \alpha 1 \), with 53 amino acids in its cytoplasmic region; and GMR\( \alpha 2 \), identical to \( \alpha i \) except that a 35-amino acid segment rich in serine and proline residues substitutes for the C-terminal 25 amino acids.\(^{2}\) Although the common membrane proximal cytoplasmic domain of the GMR\( \alpha 1 \) and GMR\( \alpha 2 \) isoforms is essential for cell proliferation,\(^{4}\) other evidence suggests that the entire cytoplasmic domain of the \( \alpha \) chain is involved but not essential, because cells that coexpressed a GMR\( \alpha \) cytoplasmic truncation mutant and \( \beta \)c showed a growth delay but were eventually responsive to human GM-CSF.\(^{5}\) The two transmembrane isoforms bind hGM-CSF with identical low affinity, and cell proliferation was observed in a murine factor-dependent cell line, FDCP-1.\(^{2,31}\) However, when GMR\( \alpha 2 \) alone is expressed in Ba/F3, cell growth is not induced despite similar low-affinity binding.\(^{6,5}\) The discrepancy between nE\( \alpha \) and cE\( \alpha \) in Ba/F3 suggests two possibilities: (1) the \( \alpha \) chain can function in certain circumstances and mediate the signal by itself or (2) the observed growth signal in Ba/F3-cE\( \alpha \) is cotransduced by an additional protein. Because one of the candidates for an accessory molecule that cooperates with cE\( \alpha \) is mu\( \beta \), we tested its role by using CTLL-2 instead of Ba/F3. Interestingly, expression of cE\( \alpha \) did not induce cell proliferation in CTLL-2, but coexpression of mu\( \beta \) reconstituted the proliferation pattern obtained in Ba/F3. These results show that cE\( \alpha \) required mu\( \beta \) to generate a constitutive growth signal. Neither extracellular high-affinity binding (nE\( \alpha \)) nor constitutive dimerization (cE\( \alpha \)) conferred proliferative potential to the \( \alpha \) cytoplasmic tail.

What region(s) of cE\( \alpha \) interacts with mu\( \beta \)\( _c \)? Because a cytoplasmic truncated cEpoR mutant that has only 19 amino acids in the cytoplasmic domain did not induce proliferation in Ba/F3 (data not shown), it is likely that the \( \alpha \) tail of the hybrid receptor is important for mu\( \beta \) to generate a signal. It is known that the ligand-induced cell proliferation requires the interaction of the cytoplasmic tails of both \( \alpha \) and \( \beta \) in

**Fig 6.** Surface expression of hybrid cytokine receptors in CTLL-2. Wild-type and transfectants were incubated with purified rabbit anti-N-terminus EpoR IgG (W) or nonspecific rabbit anti-IgG (B). The cell surface immunofluorescence was measured by FACS.

Relative Fluorescence
the high-affinity GMR complex. In the case of cEα, the observed region was responsible for the constitutive interaction of cEα and muβc. When the human βc chain instead of muβc was coexpressed with cEα in CTLL-2, factor-independent cell growth was not reconstituted (data not shown). This observation supports the idea that the murine βc chain associates with the murine extracellular cEpoR rather than the cytoplasmic human GMRα. Indeed, comparison of the extracellular sequences of human βc and muβc show that the murine but not the human protein contains an additional cysteine (C391) in strand F’ of the membrane proximal subdomain that comprises 7 β strands. It is tempting to speculate that a constitutive heterodimer is formed between the A’ β strand of cEα (R129C) and this C391 of muβc. Experiments are underway to test this hypothesis.

Does the β subunit exist as an accessory component in the intact EpoR system for either proliferation or differentiation? Previously, it was shown that EpoR induced CTLL proliferation in response to Epo. On the other hand, others showed that EpoR was not functional in CTLL leading to speculation that clonal variation of the expression of an unidentified protein plays an important role in growth signal transduction through EpoR. In our hands, neither nEpoR nor cEpoR required the β chain for their proliferation signal, as shown in CTLL-2 experiments (data not shown). It was recently reported that hybrid receptors consisting of the extracellular domain of the EpoR and either intracellular IL-3Rβ or IL-2Rβ induced proliferation, tyrosine phosphorylation, and erythroid-specific gene expression in response to Epo. The set of phosphorylated proteins and erythroid genes induced (GATA-1, SCL, α-globin, and β-globin) was similar to the nEpoR-induced pattern, suggesting the existence of an accessory protein in differentiation induction through the EpoR. It is possible that one of the additional proteins is the β chain. In contrast, none of the hybrid receptors reported here showed any capacity to induce β-globin mRNA accumulation (data not shown). These data argue in favor of the hypothesis that it is the intracellular and not the extracellular domain of the receptor that dictates the differentiation response.

Previous studies suggested that IL-3 activates JAK2 but not JAK1 in DA-3 cells. These experiments were conducted with an antibody that failed to immunoprecipitate activated, tyrosine phosphorylated JAK1. Our results show that murine IL-3 activates both JAK1 and JAK2 in Ba/F3 cells. The JAK1 antibody was shown to be monospecific, but the JAK2 antibody recognized JAK2 and Tyk2. However, Tyk2 is only activated by interferon α, β, and γ. Our results show that IL-3 activates both Jak1 and JAK2 in Ba/F3-cEα cells. We have also shown that IL-3 activates JAK1 in DA-3 and FDCP-1 cell lines (data not shown). An earlier study proposed that cytokine-specific signaling is mediated by the extracellular domain of the cytokine receptor. Our data suggest that association of JAK kinases is mediated by specific sequences in the BOX1 and BOX2 regions of cytokine receptors and not dictated by the extracellular domain of the hybrid receptor.

In conclusion, we show here that constitutive homodimerization of the cytoplasmic domain of β, mediated by extracellular R129C EpoR can confer factor-independent proliferation in two different factor-dependent cell types. The R129C EpoR mutation, when linked to GMRα, can also indirectly confer factor-independent properties, probably by forming heterodimers with murine βc. In addition, we show that both JAK1 and JAK2 are activated by the functional hybrid receptors.

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